

# Alteration of sarcoplasmic reticulum $\text{Ca}^{2+}$ ATPase expression in lower limb ischemia caused by atherosclerosis obliterans

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Atherosclerosis is a disease caused by a build-up of fatty plaques and cholesterol in the arteries. The lumen of the vessels is obliterated resulting in restricted blood supply to tissues. In ischemic conditions, the cytosolic  $\text{Ca}^{2+}$  level of skeletal muscle may increase, indicating the alteration of  $\text{Ca}^{2+}$  removal mechanisms.  $\text{Ca}^{2+}$  is transported from cytosol into the sarcoplasmic reticulum by  $\text{Ca}^{2+}$  ATPase (SERCA), with its 1a isoform expressed in adult, while its 1b isoform in neonatal and regenerating fast-twitch skeletal muscle. To investigate the role of these isoforms in ischemic skeletal muscle, biopsies from *musculus biceps femoris* of patients who underwent amputation due to atherosclerosis were examined. Samples were removed from the visibly healthy and hypoxia-affected tissue. Significantly increased SERCA1a expression was detected under the ischemic conditions ( $246 \pm 69\%$ ;  $p < 0.05$ ) compared with the healthy tissue. Furthermore, the ratio of SERCA1a-positive fibers was slightly increased ( $46 \pm 4\%$  in healthy tissue and  $60 \pm 5\%$  in ischemic tissue;  $p > 0.05$ ), whereas SERCA2a did not change. In addition, in primary cultures derived from hypoxia-affected tissue, the diameter and fusion index of myotubes were significantly increased ( $30 \pm 1.6 \mu\text{m}$  vs.  $41 \pm 2.4 \mu\text{m}$  and  $31 \pm 4\%$  vs.  $45 \pm 3\%$ ;  $p < 0.05$ ). We propose that the increased SERCA1a expression indicates the existence and location of compensating mechanisms in ischemic muscle.

**Keywords:** atherosclerosis, amputation,  $\text{Ca}^{2+}$ , skeletal muscle, SERCA1

## Introduction

Atherosclerosis is a disease related to the alteration of the endothelium (intima), with the accumulation of white blood cells. Furthermore, smooth muscle cells migrate to the intima facilitating the formation of cholesterol rich lipid plaques in the arteries (11). Atherosclerosis obliterans typically develops in large- and medium-sized arteries in the lower extremities, and the development requires several years. Skeletal muscle is largely sensitive to ischemia since this tissue represents the primary mass in the extremity. Arterial occlusion in the limbs has great clinical significance due to its high mortality and complication rates. The disease is clinically defined as chronic ischemic rest pain, ulcers, or gangrene. Mainly in aged patients, atherosclerosis could often be associated with arteriosclerosis, indicating the thickening of

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vascular smooth muscle (media) being responsible for the rigidity of blood vessels. In irreversible stage (cyanotic limb and sepsis in most serious cases), only the amputation is curative, location of which (femoral or crural) is determined by angiography showing the extent of the occlusion. In case of femoral amputation, the *arteria femoralis* is still capable of ensuring proper blood circulation, which is indispensable to healing of the stub. On the other hand, the circulation in the *arteria poplitea* and distal arteries is insufficient. Progression of the process can lead to clinical conditions in which the damage of the skeletal muscle precedes that of the vascular system pointing out the importance and sensitivity of muscle in maintaining healthy functions (1).

Skeletal muscle indicates pathological changes in several ways, e.g., chronic hypoxia induces a shift from slow oxidative to fast glycolytic fiber type in the rat diaphragm including the shift from slow sarcoplasmic–endoplasmic reticulum  $\text{Ca}^{2+}$  ATPase (SERCA2a) to the fast-type isoform, SERCA1 (9). However, no fiber-type shift was reported in rat soleus under ambient hypoxia, but the oxidative capacity of the muscle has been altered (2). SERCA1 has two muscle-specific isoforms, such as the adult type SERCA1a and the neonatal SERCA1b [recently reviewed by Zádor and Kósa (14)]. The neonatal isoform is a marker of adult muscle regeneration (14), whereas the adult isoform is an indicator of hypoxic condition (9). Altered SERCA1a expression might indicate compensatory mechanisms in ischemic conditions for maintaining the normal cytosolic  $\text{Ca}^{2+}$  concentration and reducing the  $\text{Ca}^{2+}$  overload in ischemia. Transgenic expression of SERCA1a in the heart will provide cardioprotection by reducing intracellular  $\text{Ca}^{2+}$  overload (12). Therefore, it is of interest how these  $\text{Ca}^{2+}$  pumps are expressed in muscles of ischemic human lower limbs to better enlighten the adaptation mechanisms.

## Materials and Methods

### Patients

A total of 13 patients (six males, average age  $72 \pm 4$  years and seven females, average age  $68 \pm 6$  years) who underwent femoral amputation for atherosclerosis and accepted to participate in this study were included. Patients were classified as Fontaine stage IV. The study was approved by the Ethics Committee of the Health Science Council, Budapest, Hungary [Certification number: 7917-1/2013/EKU (113/2013)]. A detailed clinical analysis was obtained from each patient recording age, diagnosis, etiology, risk factors, and medication (see Table I).

### Muscle biopsy

Skeletal muscle biopsies of about  $20 \text{ cm}^3$  [from the *musculus (m.) biceps femoris*] were obtained from the amputated limb after surgery. Tissue samples were removed from the healthy area at the amputation incision where the blood circulation was still found to be proper for wound healing, as indicated by the significant leakage of blood (see Fig. 1a, left) and from the ischemic region (10–12 cm distal) evaluated based on color, temperature changes, and insufficient blood supply (Fig. 1a, right), which was thus regarded to be a more serious ischemic tissue environment. The samples were divided into two sections that were placed into formalin and Hank's solutions. Considering that causative lifestyle and genetic factors frequently lead to the development and progression of long-term atherosclerosis, it should be emphasized that there might also be alterations in the healthy tissue, which is less affected by ischemia.

Table I. Detailed clinical analysis of the patients

No.	Gender	Age (years)	Diagnosis
1	Male	82	Atherosclerosis obliterans, gangraena pedis, ischemia cruris, hypertension, cardiomyopathia, ischemia cerebri, and insufficiencia renis
2	Male	84	Atherosclerosis obliterans and hypertension
3	Female	82	Atherosclerosis obliterans, gangraena pedis et cruris dextra, anemia, hypertension febrilis, and hypoproteinemia
4	Female	82	Atherosclerosis obliterans, gangraena cruris lat. dextra hypertension, post acute myocardial infarction (AMI), and cardiomyopathia diabetes mellitus
5	Male	64	Gangraena pedis dextra, hypertension atherosclerosis obliterans, and ischemia cerebri
6	Female	85	Gangraena pedis et cruris atherosclerosis obliterans and hypertension
7	Male	75	Atherosclerosis obliterans, ischemia pedis et cruris, hypertonia cardiomyopathia, and ulcus pylori
8	Male	66	Angina pectoris, atherosclerosis obliterans, and ulcus ventriculi
9	Female	58	Atherosclerosis, ischemia extremitas inf. hypertension, and diabetes mellitus
10	Male	63	Atherosclerosis and ischemia extremitas inferior
11	Female	61	Ischemia, atherosclerosis obliterans, gangraena digiti hypertension, and hyperlipidemia
12	Female	64	Gangraena pedis dextra, hypertension atherosclerosis obliterans, and ischemia cerebri
13	Female	44	Gangraena pedis et cruris atherosclerosis obliterans and hypertension

Biopsy location was *m. biceps femoris*, and the obstructed blood vessels are *arteria poplitea*, *arteria tibialis anterior*, and *arteria dorsalis pedis* in all cases

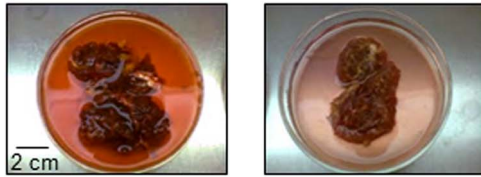
### *Preparation of cell extracts*

Muscle biopsy was stored in Hank's solution, minced into small pieces, then disrupted in homogenization solution [20 mM Tris-Cl, 5 mM EGTA, 1 mM 4-(2-aminoethyl)benzene-sulfonyl fluoride, 20  $\mu$ M leupeptin, pH 7.4 – all from Sigma, Hungary], with glass teflon Dounce homogenizer, and by sonication on ice. The ultrasound treatment was applied twice for 15 s at 60% of maximal intensity by the ultrasonic homogenizer 4710 (Cole-Parmer Instrument, Vernon Hills, IL, USA). Protein content of the samples was measured by a modified bicinchoninic acid protein assay (Pierce, Rockford, IL, USA) using bovine serum albumin (BSA) as a standard.

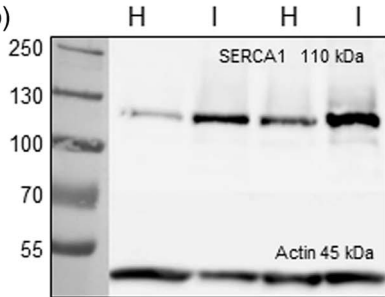
### *Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis*

Skeletal muscle tissue lysates were examined by Western blot analysis. The samples for SDS-PAGE were prepared by the addition of one-fifth volume of 5-fold concentrated electrophoresis sample buffer (310 mM Tris-HCl, pH 6.8; 10% SDS, 50% glycerol, 100 mM dithiothreitol, 0.01% bromophenol blue) to cell lysates and heated for 5 min at 80 °C.

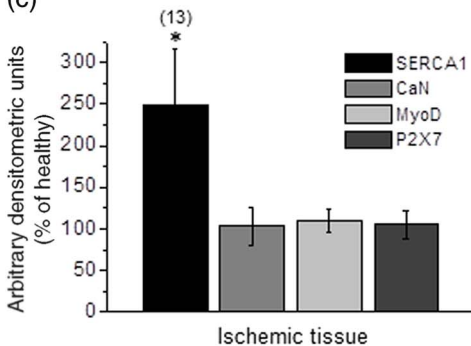
(a)



(b)



(c)



*Fig. 1.* Examination of SERCA1 expression in human lower limbs. (a) Healthy tissue from *m. biceps femoris* with sufficient blood perfusion (left panel) and ischemic tissue, 10–12 cm distal from amputation incision, viable but with color and temperature changes (right panel). Note the blood diffusion into the solution from the sample. (b) Representative experiment of the 110 kDa SERCA1 isoform detected by Western blot from total protein samples (30 µg in each lane). Healthy (H) and ischemic (I) tissues of each patient were compared (two patients shown). Actin was applied as a control. (c) Quantified expression of SERCA1, CaN, MyoD, and P2X7. Asterisk indicates significant ( $p < 0.05$ ) difference of ischemic tissue as compared with the healthy tissue. Number in parentheses indicates the number of patients. Representative data of three independent experiments are shown. Average values were calculated in case of each patient

A amount of 30 µg of protein was separated by 7.5% SDS-PAGE for immunological detection of examined proteins. The samples were electrophoretically transferred to nitrocellulose membranes (Bio-Rad, Wien, Austria). After blocking with 5% non-fat dry milk in phosphate-buffered solution (PBS), membranes were incubated with the primary antibodies at 4 °C overnight (see Table II). After washing thrice for 10 min with PBS supplemented with 0.1% Tween 20, membranes were incubated with a secondary antibody: horse radish peroxidase (HRP)-conjugated goat anti-rabbit or anti-mouse IgG, depending on the host of the primary antibody (Bio-Rad, Hungary) in 1:1,000 dilution in PBS containing 5% non-fat dry milk for 1 h. Signals were detected by enhanced chemiluminescence reaction (Thermo Scientific, Budapest, Hungary). Average values of three independent experiments were calculated for each patient.

SERCA1b lacks an exon in the 3' coding region resulting in a frameshift. SERCA1b has a longer and distinct C-terminus. Accession number is NM\_004320 for SERCA1a and NM\_173201 for SERCA1b. The SERCA1 antibody recognizes an epitope between amino acid residues 506 and the C-terminus of rabbit skeletal muscle ATPase, a region that is

*Table II.* List of antibodies used for the detection of different proteins playing important role in  $\text{Ca}^{2+}$  homeostasis and differentiation of skeletal muscle

Name	Commercial supplier	Catalog number	Host	Source	Dilutions	Epitope
MyoD	Santa Cruz	sc-377460	Mouse	Monoclonal	1:200	Amino acids 1–318 of MyoD of mouse origin
SERCA1b	E. Zádor, University of Szeged (15)		Rabbit	Monoclonal	1:800	Terminal octamer of rat SERCA1b
SERCA1	Thermo Scientific	MA3-912	Mouse	Monoclonal	1:1,000	Amino acid 506-C-terminus of rabbit skeletal muscle ATPase
SERCA2a	F. Wuytack, University of Rotterdam		Rabbit	Polyclonal	1:20,000	Amino acids 989–997 of pig SERCA2a isoform
CaN	Cell signaling	2614S	Rabbit	Polyclonal	1:1,000	Carboxy terminus of human CaN protein phosphatase 2B (PP2B)
P2X7	Alomone Labs	APR-008	Rabbit	Polyclonal	1:100	Extracellular epitope of mouse P2X7
Desmin	Sigma	D1033	Mouse	Monoclonal	1:500	Desmin purified from pig's stomach was used as the immunogen
Actin	Santa Cruz	sc-1616	Rabbit	Polyclonal	1:500	C-terminus of actin of human origin

exposed in native sarcoplasmic reticulum (SR). SERCA1b was custom made by Eurogentec (Seraing, Belgium) to a peptide corresponding to (NH<sub>2</sub>)-CLEDPEDERRK-(COOH).

### *Immunohistochemistry*

Protein expression of SERCA1 was analyzed on paraffin-embedded sections from skeletal muscles (five patients for healthy and ischemic tissue). The samples were deparaffinized using xylol and rehydrated with alcohol (100%, 96%, 70%, and 50%), and finally with PBS, pH 7.3. For hematoxylin staining, the sections were washed with deionized water, and then in 1% acid alcohol (1% HCl in 70% alcohol) for 5 min. Washing with deionized water was repeated twice. Following the staining in 1% eosin for 10 min, slides were washed in tap water for 1–5 min, dehydrated in increasing concentration of alcohols, and cleared in xylene, then slides were mounted with medium.

For SERCA1 staining, following antigen retrieval (which is applied to make the epitopes available for antibodies following formalin fixation, where protein cross-links are formed and antigen sites are masked in citrate buffer, pH 6.0, maintaining 100 °C for 10 min), slides were first incubated with the primary antibody for 1 h. After washing with PBS, sections were incubated with biotinylated anti-mouse IgG at 37 °C for 30 min (1:200; Vector Laboratories, Burlingame, CA, USA) and then by a streptavidin–HRP conjugate for 30 min. Then,

3,3'-diaminobenzidine (DAB) reagent was applied for 5 min. The sections were counterstained by hematoxylin (Sigma).

#### *Primary cell culture of human skeletal muscle satellite cells*

The procedure for obtaining satellite cells from the skeletal muscle biopsies and growing myotubes from the satellite cells has been described elsewhere (3). In brief, the muscle biopsy was dissociated at 37 °C using collagenase (Type II, Sigma, St. Louis, MO, USA) and trypsin (Difco, Detroit, MI, USA) in a  $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free PBS. After filtration and centrifugation, the pellet was resuspended in Ham's F-12 growth medium (Sigma) supplemented with 5% fetal calf serum (FCS), 5% horse serum (HS), 2.5 mg/ml glucose, 0.3 mg/ml glutamate, 1.2 mg/ml  $\text{NaHCO}_3$ , 50 U/ml penicillin, 50 µg/ml streptomycin, and 1.25 µg/ml fungizone (TEVA, Debrecen, Hungary). The cells were seeded onto sterile coverslips (32 mm diameter, 0.07 mm thick; Biophysical Technologies, Sparks, MD, USA) and maintained in a 5%  $\text{CO}_2$  atmosphere at 37 °C. After 5 days in culture, the medium was changed to Dulbecco's modified Eagle's medium (DMEM) (Sigma) supplemented with 2% FCS and 2% HS to facilitate myoblast fusion and differentiation.

#### *Immunocytochemistry*

Cultured cells were fixed with 100% methanol at -20 °C for 15 min, washed with ice-cold PBS (10 mM  $\text{NaH}_2\text{PO}_4$ , 137 mM NaCl, 2.7 mM KCl, 1.8 mM  $\text{KH}_2\text{PO}_4$ , pH 7.4), permeabilized with 0.1% Triton X-100 in PBS for 10 min, and blocked with 1% BSA diluted in PBS (blocking solution) for 30 min at room temperature. The cells were then incubated for 4 h at 4 °C with the anti-desmin primary antibody (dilution was 1:500 in blocking solution). Then, fluorescein isothiocyanate (FITC)-labeled anti-mouse secondary antibody was applied for 1 h at room temperature. Vectashield mounting medium with 4',6-diamidino-2-phenylindole (DAPI) (Vector Laboratories) was used for visualization of nuclei. Images were taken using LSM 510 META confocal microscope (Zeiss, Oberkochen, Germany).

#### *Fusion index*

The degree of differentiation was determined by calculating the fusion index, i.e., the ratio of nuclei in multinucleated cells to the total number of nuclei on 10<sup>th</sup> day of culturing (5).

#### *Statistical analysis*

Averages were expressed as mean  $\pm$  standard error of the mean. Data represent three independent experiments. Student's *t*-test was used for statistical analysis. The threshold for statistically significant differences, when comparing data with healthy, was set at  $p < 0.05$ .

## **Results**

#### *Effect of chronic ischemia on the expression of proteins involved in $\text{Ca}^{2+}$ homeostasis and development*

Skeletal muscle biopsies of about 20 cm<sup>3</sup> (from the *m. biceps femoris*) were obtained from the amputated limb after the surgery. The tissue samples were taken from the healthy and ischemic regions (10–12 cm distal, Fig. 1a). Hypoxia can be characterized by decreased ATP level and anaerobic glycolysis. Due to the impaired  $\text{Na}^+/\text{H}^+$  antiport, the subsequently increased  $[\text{Na}^+]$ , results in elevated  $\text{Ca}^{2+}$  level (10), which might be partially compensated by

SERCA1. To investigate the possible compensatory overexpression of SERCA1a – the adult SR ATPase isoform – in the ischemic tissue, samples taken from 13 patients were compared using pan SERCA1- and SERCA1b-specific antibodies. Intensity of selected bands were measured and normalized to that of actin. In all cases, the SERCA1 intensity in the ischemic tissue was compared with the intensity measured in the healthy region of the same patient. Quantitative analysis of the Western blots confirmed a very pronounced (2.46-fold) increase of SERCA1 expression in the ischemic tissue as compared with the healthy tissue (Fig. 1b). On the other hand, as the neonatal SERCA1b isoform was not detectable in immunoblots of any of the samples (data not shown), the SERCA1 band probably corresponded to SERCA1a. SERCA2a Western blot was also performed in the samples of three patients. In these samples, where previously increased SERCA1 protein expression could be detected, the slow-twitch-related SERCA2a was not altered ( $102 \pm 16\%$  of the healthy;  $p > 0.05$ , data not shown). Further tests for SERCA2a are ongoing. The samples were further examined and despite the presumed changes in  $\text{Ca}^{2+}$  homeostasis, the  $\text{Ca}^{2+}$ -dependent protein phosphatase calcineurin (CaN) expression did not show any alteration in the ischemic tissue. Expressions of the transcription factor MyoD and P2X7 purinergic receptor were independent from changes in SERCA1a expression (Fig. 1b and c). This latter result indicates that apoptotic changes were probably not induced by ischemia (8).

#### *Effect of chronic ischemia on histological alterations in m. biceps femoris*

After hemotoxylin and eosin (H&E) staining, the ischemic tissue showed a subjective increase in the amount of connective tissue as compared with the healthy region within the *m. biceps femoris* (Fig. 2a and b). These necrotic changes were also observable in SERCA1 immunostaining. Furthermore, the ratio of SERCA1-positive fibers in the total number of identified fibers, corresponding to fast twitch or may be to intermediate fiber types, increased due to ischemic conditions compared with the ratio detected in the healthy tissue (Fig. 2c and d). Nevertheless, this alteration albeit present was less pronounced ( $46 \pm 4\%$  in the healthy and  $60 \pm 5\%$  in the ischemic tissue;  $p = 0.06$ ) as compared with the expression changes of SERCA1a.

#### *Alteration in the development and morphology of primary myotubes*

The development of satellite cells of primary cultures prepared from healthy and ischemic tissues was monitored. Following the digestion of muscle biopsies, primary muscle cultures could only be generated from samples of four patients. In the other cases, either only fibroblasts could be identified – probably due to the lack of satellite cells in the cultures – or despite the presence of antibiotics in the culturing medium, the endogenous bacterial infection of tissues proved to be lethal for the cells. Muscle-specific desmin antibody was applied to identify the skeletal muscle cells and multinucleated myotubes. During muscle differentiation, myotubes develop from the fusion of myoblasts originated from precursor satellite cells. In this process, the nuclei content and the size of the myotubes show a time-dependent increase. After 10 days of differentiation, the diameter of the individual myotubes was significantly increased in cultures prepared from hypoxia-affected tissues as compared with the cells derived from the healthy tissues ( $41 \pm 2.4 \mu\text{m}$  and  $30 \pm 1.6 \mu\text{m}$ ,  $p < 0.01$ ,  $n = 53$  and 43, respectively). Similarly, fusion index was higher in cultures from ischemic environment ( $45 \pm 3\%$ ,  $n = 20$  fields of view) compared with cells prepared from the healthy region ( $31 \pm 4\%$ ,  $n = 20$  fields of view,  $p < 0.05$ ) on the 10<sup>th</sup> day of differentiation (Fig. 2e–g).



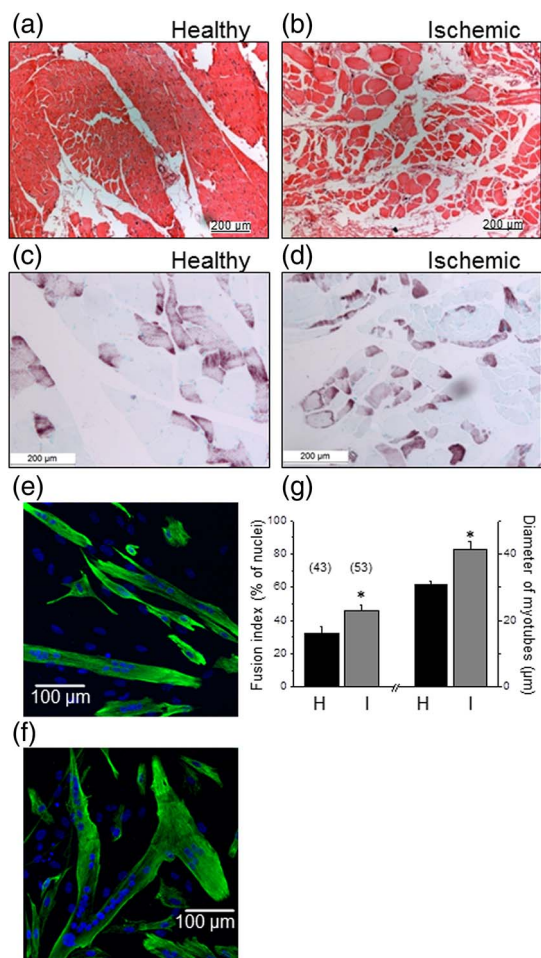


Fig. 2. Histopathological evaluation of human *m. biceps femoris*. Cross section of H&E stained fibers from healthy (a) and from ischemic (b) regions showing the necrotic changes due to ischemic conditions.

Magnification: 150 $\times$ . SERCA1-specific labeling of formalin-fixed tissue excised from healthy (c) and ischemic (d) regions, demonstrating a larger ratio of fast-twitch fibers. Magnification: 150 $\times$ .

Immunocytochemical staining demonstrating the morphological changes in differentiated human primary myotubes derived from healthy (e) and ischemic (f) tissues after 10 days of differentiation. Muscle-specific desmin was detected and visualized with FITC-conjugated secondary antibody. Nuclei were stained with DAPI. Images were recorded from 1- $\mu\text{m}$ -thick optical slices. Original magnification was 40 $\times$ . (g) Quantitative parameters of differentiated multinucleated myotubes. The fusion index and the diameter of myotubes were calculated. Number in parentheses indicates the number of identified myotubes on three different coverslips. In each culture, 20 fields of view were examined. Asterisk indicates significant ( $p < 0.05$ ) differences

## Discussion

Increased levels of SERCA1 have been demonstrated in the ischemic tissue of atherosclerotic lower limbs. The elevated expression was most likely due to SERCA1a since the other isoform, SERCA1b, was not detectable by a specific antibody. The increase of SERCA1-positive fiber number paralleled this change, but to a lesser extent than the SERCA1 level did on immunoblot. This suggested that the SERCA1 level might have been elevated within fast fibres and not only as a consequence of redistribution towards fast twitch anaerobic fibers. It is worth mentioning, however, that fiber-type grouping or central nuclei were not detected as in Ref. (7). The results also demonstrated that muscle regeneration was not initiated, rather the muscle tried to adapt through the overexpression of an adult gene type in the atherosclerotic lower limbs. Although the elevated SERCA1a protein level may not result in fully increased SR calcium pump function (6, 12), it may potentially contribute to the adaptation in postischemic conditions as seen in the rat diaphragm (9). A similar increase of SERCA2b has been observed in hind limb of ischemic mouse due to the activation achieved



by the formation of glutathione adducts (13) underlying the adaptation in calcium handling in hypoxia. On the other hand, the altered expression of SERCA2a could not be detected in the three patients examined. SERCA2a expression is indeed regulated inversely than SERCA1a in many situations in skeletal muscle, because at least the transcription of SERCA1 seems to be a “default” when SERCA2a is downregulated (14). SERCA2a is slow fiber-specific and in case of slow fiber damage, the level of SERCA1a is upregulated (even if SERCA2a is not downregulated). On the other hand, the signs of the build-up of a regenerating potential were noticeable when primary muscle cultures could be started from the biopsies (4 out of 13 patients). In such cases, the fusion index and the diameter of myotubes were higher in cultures derived from the ischemic regions compared with those from the healthy regions of muscles. However, the manifestation of the regeneration potential, as it was indicated by the lack of SERCA1b (14), is probably hampered by the ischemic condition of adult muscle tissue developed by the obliterated atherosclerotic vessels (4). Chronic limb arterial occlusion has a great clinical significance due to its complication rates. After long-term occlusions, however, it is very difficult to set up a diagnosis due to the lack of exact criteria. In irreversible stage, only amputation is curative, whereas in reversible conditions, revascularization can be applied. On the other hand, long-term ischemia results in the injury of skeletal muscle fibers. The muscle is expected to respond to reduced blood supply. Altered mitochondrial function and gene expression are supposed to participate in the adaptation processes to the restricted oxygen and nutrient conditions. The visible symptoms, such as atrophy, lack of hair, cyanotic limb, and rest pain, are direct indications of vascular dysfunction, and the affected genes and proteins in skeletal muscle might be targets for drug and gene therapy to improve the physiological conditions of the muscle, when the ischemic state is still reversible.

The SERCA, in particular, the adult fast-twitch isoform – SERCA1a, showed increased expression that could be a compensatory alteration for ischemia in human muscle, indicating the presence of adaptation mechanisms under hypoxic circumstances.

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